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Analiza antiapoptotskog proteina bcl-2 u skvamocelularnom karcinomu usne regije

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Analysis of the anti-apoptotic protein bcl-2 in oral squamous cell carcinoma

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KRATAK SADRŽAJ

Cilj *ove studije bio je analiza prisustva antiapoptotskog proteina bcl-2 u skvamocelularnom karcinomu usne regije i procena njegove eventualne uloge u razvoju i progresiji ove vrste tumora.*

Materijal i metode: *Na uzorku od 28 parafinskih blokova skvamocelularnog karcinoma usne regije, imunohistohemijskom metodom ispitan je ekspresioni status bcl-2 proteina. Mikroskopskom analizom i primenom softvera Ozaria određen je procenat imunoreaktivnih ćelija u pozitivno obojenim tumorskim regijama.*

Rezultat**:** *Pozitivnu imunohistohemijsku obojenost pokazalo je 19 od 28 uzoraka (68%) i to: 11 je bilo sa niskim (+), 4 sa srednjim (++) i 4 sa visokim procentom (+++) obojenih ćelija. U grupi pacijenata niskog stadijuma (T2) 50 % uzoraka tumora je pokazivalo ekspresiju bcl-2 proteina dok je u višim stadijumima (T3 i T4) pozitivnih uzoraka bilo 67%. Postojao je trend porasta broja ćelija sa pozitivnom bcl-2 obojenošću kod tumora u višim kliničkim stadijumima, ali ne i povećan nivo ekspresije bcl-2 proteina.*

Zaključak**:** *Oba parametra, prisustvo bcl-2 obojenosti i procenat ćelija sa bcl-2 imunoekspresijom, mogu predstavljati dopunske prognostičke parametre koji ukazuju na povećan proliferativni potencijal tumora.*

Ključne reči: bcl-2 protein, apoptoza, skvamocelularni karcinom usne regije

SUMMARY

Aim**:** *The aim of this study was to analyze the presence of the anti-apoptotic protein bcl-2 in oral squamous cell carcinoma and determine its potential role in the development and progressioin of this type of tumor.*

Materials and methods: *The expression of bcl-2 was determined in 28 paraffin blocks of oral squamous cell carcinoma using the immunohistochemical method. The percentage of the immuno-reactive cells in positively stained tumor regions was determined using the microscopic analysis and Ozaria software.*

Results**:** *Positive immunohistochemical test was observed in 19 out of 28 samples (68%) as follows: in 11 samples there was a low (+), in four a moderate (++) and in the last four a high percentage (+++) of stained cells. In the group of patients at the low stage of the disease (T2), 50% of tumor samples showed bcl-2 protein expression whereas in the higher stages (T3 and T4) of positively stained samples, this percentage was 67%. There was a trend of an increasing number of cells with positive bcl-2 staining in the tumors of higher clinical stages but not the level of bcl-2 protein expression.*

Conclusion**:** *Both parameters, the presence of bcl-2 staining and the percentage of cells with bcl-2 immunoexpression, may act as additional prognostic parameters that indicate an increased proliferative tumor potential.*

Keywords: bcl-2 protein, apoptosis, oral squamous cell carcinoma

Skvamocelularni karcinom (SCK) predstavlja najčešći tip maligniteta usne regije, a odlikuju ga brz rast, rane metastaze i učestali recidivi. Na osnovu podataka Instituta

Oral squamous cell carcinoma (OSCC) is the most frequent oral malignancy, characterized by rapid proliferation, early metastasis and frequent recidivisms. Based on

za zaštitu zdravlja Srbije »Dr Milan Jovanović-Batut« u periodu od 1990 i 2001 godine, zabeležen je dramatičan porast malignih oboljenja u našoj zemlji, za oko 150%. Kanceri usne regije takođe beleže porast, a čine 3% svih maligniteta.[1] Pomenuti klinički parametri SCK mogu pokazivati značajne varijacije od slučaja do slučaja čak i kod tumora istog stadijuma i histopatoloških karakteristika. Zbog toga se poslednjih decenija intenzivno traga za novim biološkim markerima koji bi pouzdanije mogli da predvide evoluciju bolesti kao i efikasnost primenjene terapije kod svakog pacijenta ponaosob.[2,3] Molekularna biologija nudi niz markera, a jedan od ključnih je protein bcl-2 koji igra važnu ulogu u kontroli ćelijske proliferacije i ćelijske smrti-apoptoze. Apoptoza je genetski determinisan proces koji ima ulogu u regulaciji broja ćelija u tkivu tokom morfogeneze i u odstranjivanju inficiranih ili ćelija sa oštećenjima potencijalno opasnim po ceo organizam. Različiti stimulusi, kao što su: UV i jonizujuće zračenje, hipoksija, onkogena ekspresija i dr. mogu aktivirati signalni mehanizam apoptoze. Bez obzira da li se radi o fiziološkim ili fizičko hemijskim induktorima apoptoze, ona predstavlja veoma precizno regulisan program autodestrukcije oštećenih ćelija.

Povećana ekspresija antiapoptotskog proteina-bcl-2 onemogućava realizaciju apoptotskog programa u ćelijama sa oštećenjima naslednog materijala i ujedno promoviše nekontrolisanu proliferaciju transformisanih ćelija.[4]

Takođe, inhibicija apoptoze indukovana fizičko-hemijskim agensima praćena je povećanom ekspresijom bcl-2 proteina i dovodi se u vezu sa negativnim odgovorom na primenjenu hemo ili radioterapiju kod brojnih solidnih tumora.[5]

Cilj ove studije je bio utvrđivanje prisustva bcl-2 onkoproteina i njegove potencijalne uloge u razvoju skvamocelularnih karcinoma usne regije.

Materijal i metode

U ovoj studiji korišćeni su parafinski kalupi tumorskog tkiva, dobijeni na Institutu za Patologiju Stomatološkog fakulteta. Ukupan broj analiziranih uzoraka SCK sa različitih lokaliteta usne regije i različitih kliničkih/histopatoloških karakteristika iznosio je 28. Podaci o pacijentima i tumorskom tkivu dati su u tabeli 1.

Sa ciljem da se ustanovi ekspresija bcl-2 proteina, mikroskopski preparati su podvrgnuti imunohistohemijskom bojenju pomoću DAKO ARK sistema. Ovaj sistem zasnovan je na modifikaciji avidin-biotin tehnike, gde biotinilisano sekundarno antitelo formira kompleks sa molekulima streptavidina konjugovanim sa peroksidazom, koja dovodi do vizuelne hemijske promene supstrata na antigen-antitelo kompleksu.

Sâm protokol predstavljali su sledeći koraci:

the data from the Institute "Dr Milan Jovanović-Batut" for the period between 1990 and 2001, there has been a dramatic increase of malignant diseases in our country, about 150%. Oral cancers have also increased and consitute about 3% of all malignancies.[1] The aforementioned parameters of OSCC may exhibit individual variations even in tumors of the same stage and histopathological characteristics. Therefore, during the last decades, there has been an ongoing search for new biological markers that could better predict the evolution of the disease as well as the efficacy of the applied therapy in each patient.[2,3] Molecular biology has offered a series of markers, with the bcl-2 protein playing an important part in the control of cellular proliferation and death – apoptosis. Apoptosis is a genetically determined process which has a role in the regulation of the number of cells during tissue morphogenesis and the elimination of infected and cells with potentially dangerous defects. Various stimuli, such as: UV and ionizing radiation, hypoxia, oncogene expression etc. may activate the signal mechanism of apoptosis. Regardless of apoptotic inductors (physiologic or physical/chemical), apoptosis is a highly precise program of the autodestruction of damaged cells.

An increased expression of the anti-apoptotic bcl-2 protein disables the apoptotic program in damaged cells and enhances the uncontrolled proliferation of transformed cells.[4]

Furthermore, the inhibition of apoptosis induced by physical/chemical agents is associated with the increased expression of the bcl-2 protein and is related to the negative response to the applied chemo- or radiotherapy in numerous solid tumors.[5]

The **aim** of this study was to determine the presence of the bcl-2 oncoprotein and its potential role in the development of (OSCC).

Materials and methods

Paraffin blocks of tumor tissue were obtained from the Institute of Pathology, School of Dentistry. In total, 28 samples of OSCC from different oral sites and with different characteristics were analyzed. The data on patients and tumor tissue are given in Table 1.

In an attempt to determine the expression of bcl-2 protein, the microscopic specimens were immuno-histochemically staining using the DAKO ARK system. This system is based on the modified avidin-biotin technique where biotinilised secondary antibody forms a complex with streptavidin molecules conjugated with peroxidase which results in a visual chemical change of the substrate on the antigen-antibody complex.

The protocol consisted of the following steps:

- tkivni isečci od 5µm adherirani su na DAKO superfrost staklene pločice; sledila je deparafinizacija u ksilolu i rehidratacija u etanolu i H_20
- inkubacijom u 10mM natrijum citratu pH 6.0. na 100ºC 20 min. izvršeno je otkrivanje antigenih mesta maskiranih usled fiksacije formalinom
- inkubacijom u 1% H_2O_2 10 min. inaktivirane su endogene peroksidaze;
- prekrivanjem pločica 3% bovin serum albuminom (BSA) 1h na sobnoj temperaturi blokirana su nespecifična mesta antigena
- nakon odstranjivanja blokirajućeg rastvora, dodato je bcl-2 primarno antitelo miša, rastvoreno u BSA, 1:50 (klon bcl-2-100, SIGMA, St. Louis, MO) i inkubirano preko noći na 4ºC
- nakon ispiranja u PBS-u, dodato je biotinilisano sekundarno antitelo zeca (DAKO, Glostry, Copenhagen, Denmark) rastvoreno u BSA, a inkubacija je trajala 30min. na sobnoj temperaturi
- nevezano sekundarno antitelo odstranjeno je ispiranjem u PBS-u, a zatim je dodat rastvor streptavidin-peroksidaze i hromogena (DAB-diaminobenzidin). Peroksidaza izaziva hemijske promene u hromogenu praćene razvijanjem braon boje.

U negativnoj kontroli, umesto sa primarnim antitelom, tkivo je inkubirano sa BSA u koncentraciji od 50µg/ml. Kao pozitivna kontrola korišćeni su normalni limfociti koji su prisutni na poprečnim presecima tumorskog tkiva (interna kontrola), a koji eksprimiraju bcl-2.

Semikvantitativna procena ekspresije bcl-2 proteina, podrazumevala je selekciju 10 vidnih polja u kojima se približno nalazi oko 1000 ćelija, sa ciljem da se odredi procenat bcl-2 obojenih tumorskih ćelija. Primenom softvera-Ozaria, izvršena je kvantifikacija vizuelnih informacija tj. određen je procenat braon obojenih ćelija. Rukovodeći se literaturnim podacima kada je u pitanju bcl-2 bojenje DAB sistemom, manje od 5% obojenih ćelija predstavlja negativnu, od 5 do 25% nisku (+), od 25% do 50% srednju (++) i preko 50% visoku (+++) obojenost. [6]

Za utvrđivanje asocijacije između kliničkih parametara i prisustva bcl-2 korišćeni su statistički testovi: Fišerov egzaktni test i χ^2 test. Nivo statističke značajnosti iznosio je $p<0.05$.

- 5 µm thick tissue sections were adhered onto the DAKO superfrost glass slides, dewaxed in xylol and rehydration in ethanol and H2O
- incubation with 5mM sodium-citrate, pH 6.0. at 100ºC for 20 min, uncovered antigen sites that were masked during formalin fixation
- endogenous peroxidase were blocked by incubation with 1% H2O2 for 10 min; slide covering with 3% bovin serum albumin (BSA) for 1h at room temperature blocked the antigen non-specific binding
- after the blocking solution was removed, the mouse bcl-2 primary antibody was dissolved in BSA and added, 1:50 (clone bcl-2-100, SIGMA, St. Louis, MO) and incubated overnight at 4°C
- after rinsing in PBS, the biotinilised rabbit antibody dissolved in BSA was added (DAKO, Glostry, Copenhagen, Denmark) and the incubation lasted for 30 min at room temperature
- unreacted secondary antibody was removed by rinsing in PBS and the streptavidin-peroxidase and hromogen solution (DAB-diaminobenzidin) was added. Peroxidase induced chemical changes in hromogen that were manifested as brown staining.

In the negative control, instead the primary antibody, tissue specimens were incubated with BSA, 50µg/ml concentration. Normal lymphocytes, present at the cross-sections of tumor tissue (internal control), were used as the positive control.

Semi-quantitative assessment of bcl-2 protein expression included the selection of 10 visual fields with approximately 1000 cells in order to determine the percentage of bcl-2 stained tumor cells. Using the Ozaria software, the quantification of visual information was conducted i.e. the percentage of brown cells was determined. Based on the literature data related to bcl-2 staining with the DAB system, less than 5% of cells was classified as negative, 5-25% as low (+), 25-50% as moderate (++) and more than 50% as high (+++) staining. [6]

In order to determine the association of clinical parameters with the presence of bcl-2, the following statistical tests were used: Fisher's exact and χ^2 test. The level of significance was set at $p<0.05$.

Rezultati

Nakon mikroskopske analize izvršena je procena imunohistohemijskog bojenja bcl-2 onkoproteina. U analiziranom uzorku, 9 preparata bilo je negativno, dok je 19 (68%) bilo pozitivno na bcl-2 i to: 11 je pokazivalo nizak, 4 srednji i 4 visoki procenat obojenosti, Tabela 1; slika 1-3.

Uočen je trend porasta pozitivne bcl-2 obojenosti kod tumora u višim stadijumima bolesti, slika 4.

Results

Microscopic analysis was followed by the immunohistochemical staining evaluation of bcl-2 oncoprotein. Nine specimens were classified as negative while 19 (68%) were bcl-2 positive: 11 showed low, 4 moderate and 4 high percentage of staining. Table 1, figure 1-3.

A trend of increasing positive bcl-2 staining was observed in tumors at later stages of the disease, figure 4.

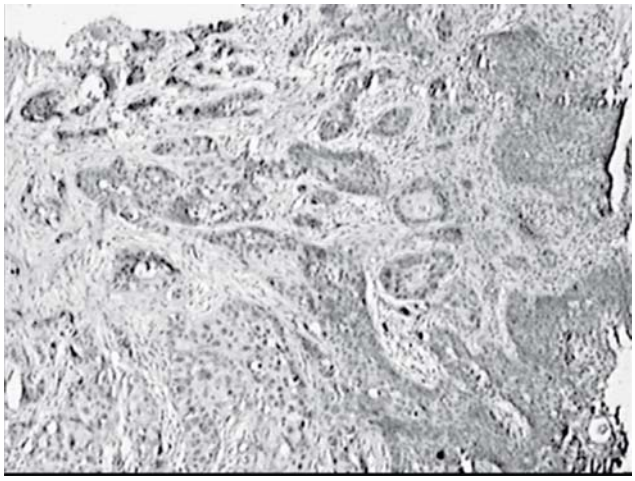
Tabela 1. Klinički i histopatološki parametri i bcl-2 ekspresija kod pacijenata sa skvamocelularnim karcinomom usne regije

Table 1. Clinical and histopathological parameters and bcl-2 expression in patients with oral squamous cell carcinomas

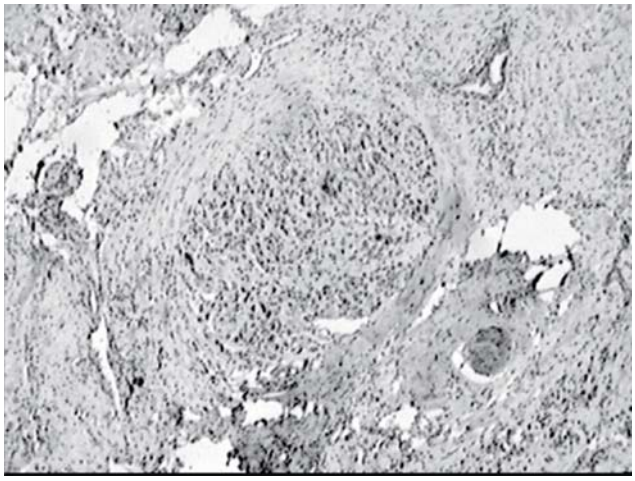
N	god.	pol	T	G	recidiv	lokalne metastaze	bcl-2	lokalitet
1.	63	M	3	3			+	pod usta
2.	62	M	2	2			+	usna
3.	48	Ž	2	2			++	jezik
4.	64	M	3	1	+		++	usna
5.	61	M	2	3			+	pod usta
6.	66	Ž	3	3	+		+	usna
7.	44	M	2	2			+++	jezik
8.	72	M	3	2			+	bukalna sluzokoža
9.	49	M	3	3			-	pod usta
10.	76	M	3	2		N1	+	jezik
11.	52	Ž	3	3			-	gingiva
12.	40	M	3	3	+	N1	+	jezik
13.	55	M	3	2			++	pod usta
14.	65	M	4	3			+	usna
15.	59	M	2	2	+		-	mandibula
16.	56	Ž	3	3	+		-	jezik
17.	69	M	3	3			++	orofaringijalni region
18.	65	M	3	3	+	N1	+	usna
19.	45	M	3	3		N1	+++	pod usta
20.	42	M	2	2	+		-	jezik
21.	59	M	3	3	+	N1	-	pod usta
22.	57	M	3	3	+		+++	jezik
23.	40	M	2	2	+	N1	-	jezik
24.	50	M	3	3		N1	-	jezik
25.	63	M	2	2			-	usna
26.	60	M	3	2		N1	+	pod usta
27.	65	M	4	2			+	usna
28.	70	M	4	2			+++	usna

T - tip invazije; G – stepen diferencijacije, gradus;
N1 – prisustvo lokalnih metastaza

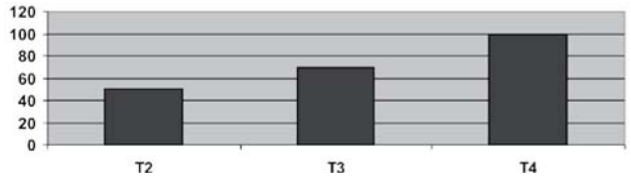
Slika 1. Tumorska regija skvamocelularnog karcinoma sa visokim stepenom bcl-2 bojenja
Figure 1. Squamous cell carcinoma with high bcl-2 staining



Slika 2. Tumorska regija skvamocelularnog karcinoma sa srednjim bcl2 bojenjem
Figure 2. Squamous cell carcinoma with moderate bcl-2 staining



Slika 3. Tumorsko ostrvo sa pojedinačno obojenim ćelijama
Figure 3. Tumor islands of squamous cell carcinoma with single cell bcl-2 staining



Slika 4. Distribucija bcl-2 pozitivnih skvamocelularnih karcinoma u odnosu na klinicki stadijum
Figure 4. Distribution of bcl-2 positive squamous cell carcinomas in relation to clinical stages

Istovremeno, nije ustanovljena razlika u pozitivnoj bcl-2 obojenosti između primarnih tumora i recidiva, karcinoma sa i bez lokalnih metastaza, karcinoma sa različitih lokaliteta i između polova. Uočen je statistički značajan porast ekspresije bcl-2 proteina kod pacijenata sa više od 60 godina (p=0.15).

Diskusija

Broj ćelija u višećelijskom organizmu striktno je regulisan putem kontrole stopa ćelijske proliferacije i programirane ćelijske smrti, tj. apoptoze. Disbalans između ova dva procesa za posledicu može da ima nekontrolisan rast ćelija sa oštećenjima u DNK molekulu, odnosno nastanak i progresiju tumora. [7]

Imunohistohemijski detektovana ekspresija bcl-2 onkoproteina kod 68% analiziranih SCK, najverovatnije ukazuje na njihov povećan proliferativni potencijal. Analizirajući literaturne podatke, pozitivna imunoekspresija u razvijenim zemljama kreće se u širokom rasponu od 7 do 70%, a slične su vrednosti i za azijski kontinent. Iako je uočen trend porasta pozitivne bcl-2 obojenosti kod viših stadijuma, što je logičan i očekivan rezultat, bitno je ukazati na njegovo prisustvo u svim kliničkim stadijumima (T2, T3, T4) što je takođe u saglasnosti sa literaturnim podacima. [8,9]

Odsustvo odnosno prisustvo antiapoptotskog proteina bcl-2 može donekle da objasni heterogenost ponašanja tumora istih histopatoloških karakteristika i kliničkih stadijuma. Drugim rečima, detektovani bcl-2 onkoprotein kod tumora koji pripadaju različitim kliničkim stadijumima, može da posluži kao dopunski prognostički marker u smislu ukazivanja na agresivnije tumorsko ponašanje. Da bi se sa sigurnošću moglo govoriti o značaju prisustva bcl-2 u SCK neophodno je duže praćenje pacijenata, odnosno toka bolesti.

Pored prisustva/odsustva pozitivne bcl-2 obojenosti, literaturni podaci ukazuju i na važnost procenta ćelija sa ekspresijom bcl-2 onkoproteina. Veći procenat ćelija sa eksprimiranim onkoproteinom pokazao se parametrom loše prognoze za niz malignih oboljenja kao što su oralni karcinomi, akutna mijeloidna leukemija, neuroblastomi i karcinomi prostate i testisa. [10,11] Zanimljiv je podatak da kod nekih tumora, kao na primer tumora dojke i jednjaka, pozitivna imunoekspresija bcl-2 predstavlja dobru prognozu.

Ipak, da se bcl-2 protein može smatrati proliferativnim markerom govori i činjenica da je ovaj protein eksprimiran u matičnim ćelijama bazalnog sloja oralne mukoze i da ima važnu ulogu u kontroli terminalne diferencijacije normalnih keratinocita.

Zaključak

Rezultati ovog istraživanja ukazuju na mogućnost upotrebe bcl-2 ekspresije kao dodatnog prognostičkog

At the same time, there was no difference in the positive bcl-2 staining between primary tumors and recidivisms, carcinoma with and without local metastasis, carcinoma from different sites and between genders. There was a statistically significant increase in bcl-2 protein expression in patients above 60 years of age (p=0.15).

Discussion

The number of cells in a multicellular organism is strictly regulated by the controlled cell proliferation and programmed cell death i.e. apoptosis. An imbalance between these two processes may result in an uncontrolled proliferation of cells with defects in the DNA molecule i.e. the development and progression of a tumor. [7]

Immuno-histochemically detected expression of the bcl-2 oncoprotein in 68% of analyzed samples of OSCC most likely indicates an increase in the proliferatory potential. Based on the literature data, the positive immunoexpression in developed countries varies greatly, between 7% and 70%, with similar values for Asia. Though there is an increasing trend of positive bcl-2 staining in later stages, which is a logical and expected result, it is important to highlight its presence in all clinical stages (T2, T3, T4), which is in agreement with literature data. [8, 9]

The absence or presence of the anti-apoptotic bcl-2 protein may, to some extent, account for the heterogenous behaviour of tumors with the same histopathological characteristics and clinical stages. In other words, the bcl-2 oncoprotein detected in tumours in different clinical stages may serve as an additional prognostic marker indicating a more aggressive tumor behavior. For more certain conclusions about the importance of the presence of bcl-2 in OSCC, a longer patient observation i.e. the course of the disease, is required.

Apart from the positive/negative bcl-2 staining, literature data also point out the importance of the percentage of cells with bcl-2 oncoprotein expression. A higher percentage of cells with the oncoprotein have shown to be the parameter of a bad prognosis for a series of malignant diseases, such as oral carcinoma, acute myeloid leukemia, neuroblastomas, and prostate and testicular cancer. Interestingly, in certain tumor types e.g. breast or epiglottis, the positive immunoexpression of bcl-2 is associated with a good prognosis.

However, the bcl-2 protein can be considered a proliferative marker because this protein is present in the stem cells of the basal layer of oral mucosa and has an important role in the control of terminal differentiation of normal keratinocytes.

Conclusion

The present results indicate the possibility of the use of bcl-2 expression as an additional prognostic parameter

parametra agresivnijeg ponašanja skvamocelularnih karcinoma usne regije.

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of a more aggressive behavior of oral squamous cell carcinoma.

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